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STUDIES ON BIOCHEMICAL MUTATIONS IN OPHIOSTOMA WITH SPECIAL REFERENCE TO SOME PYRIDOXAMINE-DEFICIENT STRAINS

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INAUGURAL DISSERTATION

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Introduction.

The present paper is, for the most part, a summary of the results from the following communications, published in Physiologia Plantarum. These are referred to in the text by I–IV. It also includes a more detailed discussion of some of the problems dealt with in the earlier papers.

I (together with N. Fries). Some new and interesting biochemical mutations obtained in Ophiostoma by selective enrichment technique. — Physiol. Plant. 5:130. 1952.

II. Effects of compounds of the vitamin B_6 group on the growth of Ophiostoma multiannulatum. — Physiol. Plant. 12:100. 1959.

III. Further studies in the physiological and genetical interrelations between pyridoxamine- and pyridoxal-deficient mutants of Ophiostoma. — Physiol. Plant. 12:412. 1959.

IV. Some further data on pyridoxamine-deficient mutants in Ophiostoma. — Physiol. Plant. 12:665. 1959.

Isolation of mutants (I).

The pioneer work in the field of biochemical genetics was performed by Beadle and Tatum (1941), who isolated mutant strains with nutritional deficiencies of the ascomycetes *Neurospora crassa* and *N. sitophila*. In *Ophiostoma* the first mutants were isolated by Fries (1945).

In the first methods used for mutant isolation the cells are transferred to a complete agar medium after the treatment with a mutagenic agent. The colonies or mycelia growing out are then examined individually as to their nutritional demands, compared with the mother type cells. Such methods for "total isolation" are labour-consuming and generally give a relatively low yield of mutants. Labour-saving enrichment techniques have therefore been devised for many organisms. Different principles have been used for the enrichment. A layer-plating technique, which takes advantage of the fact that mutants have different growth rates in minimal and enriched media, has been devised for *Escherichia coli* by LEDERBERG and TATUM (1946). This technique has also been used with some success for fungi, e.g. *Ustilago* (PERKINS 1949) and *Aspergillus nidulans* (PONTECORVO 1953). By visual inspection of ascospores on an appropriate medium, slowly growing spores (and thus mutants) may be selected for in *Neurospora* (LEIN *et al.* 1948). In such fungi, where the spores or conidia develop hyphae during germination, mutants may be concentrated by filtering techniques. This has been done with *Ophiostoma* (FRIES 1947), *Aspergillus* (HOCKENHULL 1948), and with *Neurospora* (CATCHESIDE 1954; WOODWARD *et al.* 1954). The most effective enrichment method devised until now is the penicillin technique, used for bacteria sensitive to this antibiotic (LEDERBERG and ZINDER 1948; DAVIS 1948). The antibiotic is able to kill growing bacteria while non-growing (mutants) survive. A similar "differential killing" is used in the starvation technique of PONTECORVO (1953) for *Aspergillus nidulans*. This technique is founded on the observation made in *Ophiostoma* by FRIES (1948) that mutant conidia are often more viable than the conidia of the mother strain in a starvation substrate. With this method yields of up to 60 per cent mutants have been obtained.

When the present investigation was started, the supposition was made that the mutant types isolated so far in *Ophiostoma* did not represent all obtainable types. The isolation experiments were therefore planned with the aim of searching for more uncommon types of biochemical mutations in this fungus.

The isolation method used was the filtration technique devised by FRIES (1947). The procedure was modified in the course of the work (I). The complete medium used in the isolation experiments was altered in the following way: (1) The concentration of the constituents of the medium (malt extract, yeast extract, acid-hydrolyzed

casein) known to contain inhibitory substances for mutants was reduced to about 10 per cent of the amount used previously in isolation experiments in *Ophiostoma* (FRIES 1948); (2) A carefully prepared liver extract was added to the medium. The liver extract, prepared from fresh liver, was supposed to be a supply of chemically labile and heat-sensitive compounds and to contain other growth factors (vitamins, amino acids etc.) in "physiologically beneficial" proportions.

The expectations of obtaining previously unisolated mutant types were fulfilled at least in part (I). Several strains with double requirements appeared. They were probably double mutants. One of the strains, W 3, had three specific requirements (adenine, histidine, glycine). Crosses between this mutant and the wild type showed that it was a single-gene mutation. A glutathione-less strain, W 302, was also isolated. Its nutritional requirements have been described in a separate paper (WIKBERG 1954). Two of the strains, W 233 and W 366, were pyridoxamine heterotrophs (not reported in I). The physiology of these two has been studied more in detail (II, III).

The glutathione-less strain may be looked upon as a representative of a mutant type which responds to chemically labile compounds (WIKBERG 1953). The observation was made that it did not grow in stock culture tubes containing the ordinary complete medium which had been stored for some time in the laboratory before inoculation. Growth started in such tubes if supplied with glutathione. The inability of the stored medium to support growth probably depended on hydrolysis of the glutathione in the complete medium during storage.

The pyridoxamine-less strains, W 233–2 and W 366–10 (f_1 strains of W 233 and W 366, respectively), are inhibited by constituents of the complete medium. The appearance of this type in the enrichment experiment probably also depended upon the favourable composition of the medium used. The rarity of this type in ordinary isolation experiments is exemplified by the fact that only one such strain, No. 2855 (IV), has been obtained in the laboratory of Professor FRIES, in spite of the fact that tens of thousands of isolates have been studied during the past 15 years.

Physiological and genetical studies on pyridoxamine-deficient strains.

(a) The response to different forms of vitamin B_6 (II, III, IV).

Vitamin B_6 has been found in a variety of tissues both from plants and animals. Its wide distribution indicates that it is an important, perhaps indispensable, metabolite for all living cells. This holds for all the three forms pyridoxine, pyridoxal, and pyridoxamine (Rabinowitz and Snell 1948). Probably all microorganisms which do not need the vitamin for growth are able to synthesize it by themselves. Direct analysis has shown that this is the case in some bacteria (Thompson 1942), in yeast, and filamentous fungi, e.g. *Neurospora crassa* (Stokes *et al.* 1943) and *Penicillium* (Rabinowitz and Snell 1948).

In contrast to these B_6 autotrophs, many microbial species need B_6 for growth or are stimulated by it. This means that the organisms in question are unable to synthesize the vitamin as a whole or in part. In most cases, when an organism has been tested for B_6 requirement, only pyridoxine has been used. In all species of B_6-heterotrophic fungi for which the three forms of B_6 have been tested (*Ceratostomella ulmi*, *Mycoderma valida*, and *Neurospora sitophila* (Snell and Rannefeld 1945)), it was found that all three forms were interchangeable. This is not the case in certain bacteria (*Lactobacillus* and *Streptococcus sp.*) which need pyridoxamine or pyridoxal, or only pyridoxal. In fact, pyridoxamine and pyridoxal were originally detected with the pyridoxal (pyridoxamine)-deficient bacterium *Streptococcus faecalis* (for references see Williams *et al.* 1950).

From enzyme studies it is known that pyridoxine, pyridoxal or pyridoxamine themselves are not the active forms of the vitamin but pyridoxal phosphate and pyridoxamine phosphate. The former acts as a coenzyme for many enzymes: transaminases, racemases, amino acid decarboxylases, etc., while pyridoxamine phosphate participates only in transaminations. One possible role of pyridoxamine phosphate may be that it is a storage form which is easily converted to pyridoxal phosphate (Rabinowitz and Snell 1947; Beechey and Happold 1957).

The growth reactions (II, III, IV) of the mutants may be summarized as follows. Both W 233–2, W 366–10 and No. 2855 respond positively to pyridoxamine. The quantitative yield of mycelium equals that of the wild type, except for No. 2855, where more of the

Table I. Effect of pyridoxine, pyridoxal, and pyridoxamine on the growth of different pyridoxamine-less mutants and wild type at 30°C and 20°C.

0 = no growth, 1 and 2 = impaired growth, 3 = good growth.

Mutant strain	Induced by	30°C			20°		
		Pyri-doxine	Pyri-doxal	Pyridox-amine	Pyri-doxine	Pyri-doxal	Pyridox-amine
W 233–2, pyridox-amine-less	UV	0	1	3	0	—	3
W 366–10, pyridox-amine-less	UV	0	1	3	2	2	3
W 366–10–1, pyri-doxal-less	Spont. from W 366-10	0	3	3	2	3	3
No. 2855, pyridox-amine-less	X-rays	0	1	3	0	2	3
No. 51, wild type	—	3	3	3	3	3	3

vitamin must be given for the same yield. The utilization of pyridoxal is impaired in all three mutants: the growth starts after a long lag and the vitamin is active only at high concentrations (about 300 times the lowest concentration which gives appreciable growth of the wild type). Pyridoxine lacks a positive effect at 30°C. The utilization seems to be totally blocked at this temperature, since this vitamin cannot be assimilated even if growth is initiated by giving the active form pyridoxamine simultaneously.

From conidial populations of strain W 366–10, one isolate, W 366–10–1, was obtained which responds to both pyridoxal and pyridoxamine in the same manner as the wild type. However, uilization of pyridoxine was still blocked at 30°C.

At 20°C the metabolic block caused by the mutation is partially relieved for both pyridoxine and pyridoxal in strain W 366–10. Wild-type growth habit is not attained, however. No. 2855 shows a similar temperature sensitivity, but only for pyridoxal (IV). This vitamin is utilized better at 20°C than at 30°C. The pyridoxal-less strain W 366–10–1 is also temperature-sensitive. The response to pyridoxine at 20°C is quantitatively the same as in the strain from which it was derived, viz. W 366–10 (III). A summary of the growth effects is given in Table I.

Pyridoxal phosphate and pyridoxamine phosphate promote growth of both the wild type and the mutants. For all strains the vitamin phosphates were poorer vitamin sources than the unphosphorylated derivatives. The mutants W 233–2 and W 366–10 differed from wild type with their response to the phosphates. No growth occurred below 1 mμmol per 10 ml medium of pyridoxamine phosphate and 10 mμmol per 10 ml of pyridoxal phosphate. Strain No. 2855 seems to represent an intermediate type between W 233–2 and W 366–10, on one side, and No. 51, wild type, on the other. The threshold concentrations for strain No. 2855 with pyridoxamine phosphate and pyridoxal phosphate were about 0.1 and 1 mμmol per 10 ml, respectively. For No. 51 the corresponding values are 0.01–0.03 mμmol for both vitamin phosphates.

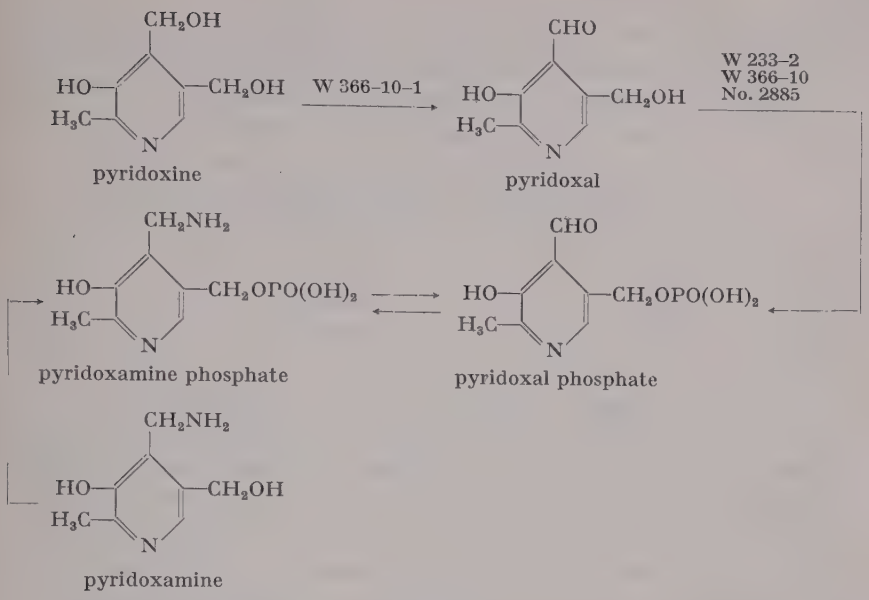
The observation made in *Ophiostoma*, that the utilization of the B_6 phosphates is impaired, does not seem to be unique for this organism. The same has been found for *Saccharomyces carlsbergensis* (Rabinowitz & Snell 1947). *Streptococcus faecalis* seems to be the only organism which utilizes pyridoxamine phosphate at the same rate as pyridoxamine. Similar results as with *Ophiostoma* have been reported for thiamine phosphates. These compounds are less efficient than thiamine as growth factors for thiamine-deficient mutants of *Neurospora* (Eberhardt 1957).

For thiamine monophosphate the evidence brought by Kiessling (1957) makes it very probable that this compound does not enter the yeast cell as an intact molecule, but is hydrolyzed before being assimilated. This may also hold for the B_6 phosphates. For *Ophiostoma*, wild type, it has been shown (II) that pyridoxamine phosphate was rapidly hydrolyzed by living conidia suspended in phosphate buffer solution.

At present it cannot be decided whether the phosphates are assimilated as intact molecules or not. However, the evidence obtained cautions one against laying too heavy an emphasis on the growth effects of these compounds when discussing the biosynthetic path of the compounds in the cell.

Pyridoxine interferes with the utilization of both pyridoxal and pyridoxamine in the mutants. A discussion and evaluation of these effects is made below.

On the basis of the growth effects the following tentative scheme for the biosynthesis of the coenzymes may be set up:



It is supposed that a partial block lies after pyridoxal in the pyridoxamine-less mutants. In the pyridoxal-less isolate a block is assumed between pyridoxine and pyridoxal.

Evidence obtained from growth experiments with other micro-organisms was compared with this scheme (II). It was found that the nutritional demands of various bacteria are not in contradiction to the biosynthetic scheme proposed.

It may, of course, be questioned whether the coenzymes, pyridoxal phosphate and pyridoxamine phosphate, are actually synthesized by *Ophiostoma*. Assay of pyridoxamine phosphate has not been made, but the synthesis of pyridoxal phosphate from pyridoxine has been shown in an experiment, performed in the following way.

A two-day-old cell suspension of *Ophiostoma*, wild type, was starved for B_6 by incubating the cells in B_6-free, minimal nutrient medium for two periods of 24 hours each. The suspension was washed with water before each incubation period. This treatment was followed by incubation at 30°C in 1/15 *M* phosphate buffer, pH 6.0, with the addition of pyridoxine to the medium (0, 10, 20 and 40 mμmol per ml). The suspension contained about 0.9 mg conidia, dry weight, per ml. After 4 hours samples were taken from each mixture. The conidia were washed, suspended in the same volume of 0.1 *M* NaOH, heated for 5 min. at 100°C, cooled, and adjusted

Table II. Synthesis of pyridoxal phosphate from pyridoxine by *Ophiostoma* No. 51, wild type. The amounts of pyridoxal phosphate are the mean of 2–4 determinations.

Pyridoxine added mμmol per ml cell suspension.	Pyridoxal phosphate μg per mg conidia, dry weight.
0	4.8
10	36.5
20	39
40	47

to pH 5 with HCl. Without removing the suspended matter aliquots of the extract were assayed for pyridoxal phosphate manometrically with tyrosine apodecarboxylase (Gunsalus and Smith, 1957). See Table II.

The *in vivo* synthesis of codecarboxylase (pyridoxal-5-phosphate) from members of vitamin B_6 has previously been demonstrated in *Saccharomyces carlsbergensis, Neurospora sitophila* (Bellamy, Umbreit and Gunsalus 1945) and in rat liver slices (Trufanov and Kirsanova 1946). After the detection of the pyridoxal kinase reaction in *Streptococcus faecalis* (Gunsalus and Bellamy 1944) the enzyme pyridoxal kinase has been found in a variety of materials, indicating its wide distribution in nature: in mouse brain (Roberts and Frankel 1951), in rat liver (Binkley and Christensen 1951), in *Escherichia coli* and baker's yeast (Hurwitz 1953). The yeast enzyme has been purified and studied for its specificity. Except for pyridoxal, it is able to phosphorylate a variety of B_6 derivatives, including pyridoxine, pyridoxamine and several antivitamins derived from B_6.

One more enzyme related to B_6 metabolism has been studied *in vitro*, viz. pyridoxamine phosphate transaminase in *Escherichia coli*. This enzyme mediates the interconversion between pyridoxal phosphate and pyridoxamine phosphate (Beechey and Happold 1957). The nature of the amino group acceptor is unknown. The conversion of pyridoxamine phosphate to pyridoxal phosphate has also been demonstrated to occur in cell-free preparations of *Clostridium welchii*. Pyruvate was the acceptor of the amino group and alanine was formed (Meister *et al.* 1951).

Pyridoxine, pyridoxal, and pyridoxamine are oxidized to pyridoxic acid and excreted in urine when fed to humans (Huff and

Perlzweig 1945). Pyridoxal was supposed to be the intermediate between pyridoxine (pyridoxamine) and pyridoxic acid. The step pyridoxine→pyridoxal and thus the enzyme pyridoxine oxidase has in fact been demonstrated in extracts from rat tissue (Braunstein and Bukin 1956).

The participation of pyridoxamine in transamination reactions has been indicated in studies with *Streptococcus faecalis* (Bellamy *et al.* 1945), *Escherichia coli* (Gunsalus and Tonzetich 1952), higher plants (Wilson *et al.* 1953), and heart muscle (Tanenbaum 1956). Whether pyridoxamine takes part in the transaminations without prior conversion to pyridoxamine phosphate is not known.

The biochemical information regarding B_6 metabolism seems yet too incomplete to permit comparisons in detail with the results from growth experiments. Pyridoxine is phosphorylated *in vitro* by yeast pyridoxal kinase (Hurwitz 1952). If the same phosphorylation reaction occurs *in vivo* the transformation pyridoxine phosphate →pyridoxal phosphate seems plausible. As yet it has not been demonstrated. Growth data from *Ophiostoma* mutants indicate that phosphorylation of pyridoxal is impaired. If this is the case the direct conversion pyridoxamine→pyridoxal seems to be excluded, at least as a primary reaction leading to pyridoxal phosphate.

(b) Antagonistic interactions between pyridoxine, pyridoxal, and pyridoxamine (II, III, IV).

Pyridoxine is not utilized by the mutants at 30°C. Furthermore, when this vitamin is given in combination with the active form pyridoxamine, growth is inhibited, and at a pyridoxine–pyridoxamine ratio of about 100:1 inhibition is total. The inhibition is of the competitive type over a wide range of concentrations. Pyridoxal interferes in the same way with pyridoxamine though total inhibition does not occur, since pyridoxal is utilized to some extent (II). With the pyridoxal-less strain, W 366–10–1, it was shown that pyridoxine, which does not support growth of this mutant at 30°C, competes with pyridoxal. The index for total inhibition is about 30:1 (III).

It may thus be noted that antagonism occurs between the different forms of the vitamin in all the possible combinations: pyridoxine–pyridoxamine, pyridoxine–pyridoxal, and pyridoxal–pyridoxamine. The wild-type strain is not inhibited by pyridoxine at least up to 10 μmol pyridoxine per 10 ml.

The interaction between pyridoxine and pyridoxamine was also

Table III. The effect of pyridoxamine and pyridoxine alone and in combination on the growth of pyridoxamine-deficient W 366-10 at 20°C in 125 ml flasks with 20 ml medium. Dry weights of mycelium in mg after 6 days.

Vitamin added	mμmol vitamin per flask				
	1	10	30	100	300
Pyridoxine	6.9	11.3	9.4	12.5	—
Pyridoxamine	51.0	53.6	—	55.8	—
Pyridoxamine 1 mμmol + Pyridoxine	—	24.3	7.2	8.4	8.5

studied at 20°C with the temperature-sensitive strain W 366–10. When both pyridoxine and pyridoxamine are given together at this temperature, pyridoxine depresses the amount of mycelia produced below that which is obtained with pyridoxamine alone. At a high concentration of pyridoxine the production then is about equal to that in the series with pyridoxine alone. Total inhibition did not occur even at high concentrations of pyridoxine at this temperature. Table III.

Antagonistic interactions of the type described in these *Ophiostoma* mutants have been observed in biochemical mutants from many different organisms. In *Escherichia coli* 5-dehydroshikimic acid competitively inhibits growth of a shikimic acid-requiring strain (Davis 1952). Guanine-deficient strains of *Ophiostoma* are inhibited by adenine and hypoxanthine (Fries 1949). Lysine-requiring strains of *Neurospora* (Doermann 1944) and *Ophiostoma* (Bergström and Sjöbeck 1950) are inhibited by arginine. Histidine-less *Neurospora* strains are competitively inhibited by arginine and methionine in combination (Mathieson and Catcheside 1953). Arginine-less mutants of *Neurospora* (Houlahan and Mitchell 1948) and *Ophiostoma* (Bergström and Sjöbeck 1950) are inhibited by lysine. Finally the isoleucine–valine antagonism in *Neurospora* (Bonner, Tatum and Beadle 1943) and *Escherichia coli* (Cohen and Rickenberg 1956) may be mentioned.

These antagonistic interrelations thus occur within different microbial species. A few common traits for the phenomenon may be noted. (1) It appears in biochemical mutants which require one or two of the compounds involved, (2) the antagonisms seem to be competitive,

(3) the inhibitory compounds involved are metabolites themselves, synthesized by the wild-type form of the organism.

It seems quite clear that the antagonisms described for the B_6 vitamins in *Ophiostoma* belong to the same category of antagonisms as are found in other biochemical mutations for other metabolites. The inhibitory effects have been explained in terms of interference with the metabolic paths in the cell (Bergström and Sjöbeck 1950; Doermann 1944). Recently, however, evidence has been brought forward for an alternative explanation. There seems to be definite proof of the existence of a permeability barrier for nutrilites in bacteria, e.g. in *Escherichia coli*. It has also been possible to identify several highly specific permeation systems in this organism, which mediate the entry of organic nutrilites into the cell and control the accumulation of these compounds within the cell (Cohen and Monod 1957). Systems which accumulate amino acids, e.g. valine, have been found. Isoleucine competes with valine for entry into the cell and is able to displace already accumulated valine. In valine-less mutants there was found a striking parallelism between the isoleucine–valine ratio necessary to displace 50 per cent of the valine accumulated and the ratio of isoleucine–valine, at which growth of the mutant was inhibited by 50 per cent. Isoleucine thus seems to prevent the uptake of valine from the medium and growth is impaired in the mutant because of internal valine deficiency (Cohen and Rickenberg 1956).

In the histidine-less mutants mentioned above (Mathieson and Catcheside 1953) histidine accumulates from the culture medium, and uptake is prevented by a combination of the inhibitors arginine and methionine. The wild type does not differ from the mutant in this respect.

As regards the antagonism reported for the B_6 vitamins, it cannot *a priori* be excluded that pyridoxine might interfere in the conversion of pyridoxamine and pyridoxal to pyridoxal phosphate or might inhibit some reaction where pyridoxal phosphate participates. The result of the experiment with the temperature-sensitive strain reported above (Table III) may be interpreted so that the inhibition is concerned either with the uptake of pyridoxamine or with some stage in the conversion of this vitamin to pyridoxal phosphate and not with the functioning of the coenzyme. In this connection some results from an investigation by Rabinowitz and Snell (1953) are relevant. In studying the effect of the B_6 antagonist, 4-desoxypyridoxine, on the

growth of various microorganisms the following results were noted for *Saccharomyces carlsbergensis* and a *Neurospora sitophila* mutant (the latter being pH-sensitive and pyridoxine-less). When *S. carlsbergensis* was cultivated under such conditions that B_6 was synthesized by the organism (that is, without the addition of thiamine to the medium), the amount of 4-desoxypyridoxine required to inhibit growth was over 150 times higher than the amount needed for this purpose under conditions of B_6 heterotrophy. For the *Neurospora sitophila* mutant the antivitamin did not inhibit growth at all when B_6 was synthesized by the fungus. As the authors suggest, one of the possibilities for the action of the antimetabolite is that it prevents the uptake of vitamin from the medium, though the experiments do not provide any conclusions as to the site of action. It is, of course, impossible to apply conclusions gained from one organism as explanations for similar phenomena in others without experimental evidence. However, in view of the studies of the antagonisms referred to above, it seems justifiable as a working hypothesis to regard the inhibitory effects of pyridoxine (and pyridoxal) in *Ophiostoma* as a competition between these metabolites and pyridoxamine at a site without direct relation to the biochemical interconversions of vitamin B_6 within the cell.

(c) Genetical interrelationships (III, IV).

Crosses between the pyridoxamine-less mutants (W 233–2, W 366–10, and No. 2855) and the wild type resulted in a progeny of wild types and mutants (III, IV). Five substrains of W 366–10 from such a cross were compared in respect of their responses to pyridoxal. The character of slow response to this vitamin passes through the sexual cycle, but minor differences in the minimal active dose required for growth in different strains were noted. These variations were interpreted as caused by 'modifying genes' in the genetic background. An influence of the genetic background on threshold requirements of the kind reported in these strains has earlier been observed in histidine-deficient mutants of *Neurospora crassa* (Mathieson and Catcheside 1953).

From intercrosses between all the mutant strains wild-type mycelia were obtained in the progeny (III, IV). In an ascospore suspension obtained from the cross No. 2855(+) ×W 366–10–14(–) the frequency of wild type was estimated as 0.8 per cent or less.

Back-mutations to prototrophy have been found in W 233–2 and

W 366–10, but not in No. 2855. In the populations studied the frequency was determined to be of the order of 10^{-8}–10^{-7} (IV). Spontaneous back-mutations are therefore probably not responsible for the occurrence of wild-type spores in the progeny from the intercrosses between the pyridoxamine-less mutants. On the basis of the present experimental evidence the most reasonable explanation of the results from the intercrosses therefore seems to be that the wild-type spores have been formed through recombination. The pyridoxamine-less mutants are thus non-allelic and the mutated genes seem to be closely linked.

Cross-feeding experiments in pyridoxine-supplemented medium between conidia of W 233–2 and W 366–10–1 did not result in growth. The experiment thus did not give support to the view that the genes in the mutants are complementary. Heterocaryon tests for non-allelism are not possible since the phenomenon of heterocaryosis is not known in *Ophiostoma* (Fries and Trolle 1947).

Strain W 366–10–1, pyridoxal-less, an isolate from a suspension of W 366–10 grown in pyridoxal-supplemented medium, was shown to be a stable variant of W 366–10. More pyridoxal-less strains, phenotypically identical with W 366–10–1, were found in a low frequency together with prototrophic reversions in a suspension of W 366–10 grown in pyridoxamine-supplemented medium. Both the pyridoxal-less and the reversions are supposed to be spontaneous mutations derived from W 366–10. From suspensions of W 233–2 no pyridoxal-less types could be found.

The pyridoxal-less isolate, W 366–10–1, was crossed to the wild type. In the progeny pyridoxal-less types were found, which proved that the character is heritable and has been formed through mutation. In addition, pyridoxamine-less types were crossed out in 1:1 proportion together with pyridoxal-less types. This finding suggested that a suppressor gene was operating. A prototrophic reversion of the pyridoxal-less W 366–10–1, supposed to carry the suppressor, was therefore combined with the pyridoxamine-less strain W 366–10. The progeny consisted of both wild-type, pyridoxal-less and pyridoxamine-less strains. The result proves conclusively that a mutation in a second gene together with the primary-gene mutation conditions the pyridoxal-less growth habit. The suppressor only restores the ability to utilize pyridoxal, while a block after pyridoxine persists in the strains at 30°C. No other phenotypic effect for the suppressor gene has been found.

Although no spontaneous pyridoxal-less mutations were ever observed in cultures of W 233–2, mating experiments showed that the suppressor was able to act upon the mutated gene of this strain, too, when the suppressing allele was transferred into the genome. This was brought about by crossing W 233–2 with a wild-type reversion of the suppressor-carrying W 366–10–1. The pyridoxal-less strains found among the progeny from this crossing were not temperature-sensitive, however, in similarity with the mother mutant W 233–2 (III).

The biochemical effect of the primary mutation to pyridoxamine heterotrophy is difficult to evaluate. The existence of a partial block between pyridoxal and pyridoxal phosphate seems to be substantiated by growth data from all three mutant strains at 30°C. Pyridoxine is thought to be converted to pyridoxal but the intracellular concentration does not exceed the threshold value required. As is the case for most biochemical mutations, where a detailed analysis of the different steps of a blocked reaction sequence has not been performed, it is generally impossible to conclude from a single mutant type if the primary block concerns several steps in the same sequence or not. On the basis of evidence from a large number of biochemical mutants analyzed in *Neurospora*, it has been supposed that, as a rule, a single-gene mutation only blocks a reaction sequence in a single step (Horowitz 1950).

The effect of the suppressor gene is difficult to evaluate in relation to the biosynthetic sequence, since the different steps of the sequence itself require further elucidation. Some possible modes of action of the suppressor have, however been suggested (III). If, in contradiction to the experience from other biochemical mutations, it is assumed that the primary mutation acts on two biochemical steps, the action of the suppressor can be explained by assuming that this gene only relieves the latter step, that after pyridoxal. Finally, a possibility not considered before is that the transformation of pyridoxine to pyridoxal phosphate is not via pyridoxal as proposed but via pyridoxine phosphate. If in addition it is assumed that all three vitamins are phosphorylated by the enzyme pyridoxal kinase, the possibility exists that qualitative differences in this enzyme brought about by the mutations might be responsible for the different mode of growth in the mutant strains. The present experimental evidence does not permit any decision between the possibilities outlined.

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